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Isolation and characterisation of mineral-oxidizing "*Acidibacillus*" spp. from mine sites and geothermal environments in different global locations

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Abstract

Eight strains of acidophilic bacteria, isolated from mine-impacted and geothermal sites from different parts of the world, were shown to form a distinct clade (proposed genus "*Acidibacillus*") within the phylum *Firmicutes*, well separated from the acidophilic genera, *Sulfobacillus* and *Alicyclobacillus*. Two of the strains (both isolated from sites in Yellowstone National Park, USA) were moderate thermophiles that oxidized both ferrous iron and elemental sulfur, while the other six were mesophiles that also oxidized ferrous iron, but not sulfur. All eight isolates reduced ferric iron to varying degrees. The two groups shared <95% similarity of their 16S rRNA genes and were therefore considered to be distinct species: "*A. sulfuroxidans"* (moderately thermophilic isolates) and "*A. ferrooxidans"* (mesophilic isolates). Both species were obligate heterotrophs; none of the eight strains grew in the absence of organic carbon. "*Acidibacillus"* spp. were generally highly tolerant of elevated concentrations of cationic transition metals, though "*A. sulfuroxidans"* strains were more sensitive to some (e.g. nickel and zinc) than those of "*A. ferrooxidans"*. Initial annotation of the genomes of two strains of "*A. ferrooxidans*" have revealed the presence of genes (*cbbL*) involved in the RuBisCO pathway for CO_2 assimilation and iron oxidation (*rus*) though with relatively low sequence identities.

1. Introduction

Acidophilic microorganisms comprise a large variety of different species that are widely distributed in all three domains of known life-forms [1]. While the greatest number (and earliest isolates) of known extremely acidophilic bacteria are members of the phylum Proteobacteria, other phyla, including the Firmicutes, Nitrospirae, Actinobacteria and Aquificae, all include species that grow optimally at pH <3. Currently, the Firmicutes (endospore-forming eubacteria with low G+C contents) include two genera of extreme acidophiles, *Sulfobacillus* and *Alicyclobacillus*, most species of which are moderate thermophiles (growth temperature optima of 40 – 60ºC) though some are mesophilic. *Sulfobacillus* spp. are typically found in mineral- and sulfur-rich acidic environments, such as solfatara fields and biomining operations, and are characterised by having far greater metabolic versatilities than many of the more specialised bacteria (such as *Leptospirillum* and *Acidithiobacillus* spp.) with which they frequently cohabit. Species such as *Sb. thermosulfidooxidans, Sb. acidophilus* and *Sb. beneficiens* can grow autotrophically by oxidizing inorganic electron donors (sulfur, ferrous iron and hydrogen) and fixing carbon dioxide, heterotrophically using organic carbon as both energy and carbon source, and chemolitho-heterotrophically whereby they obtain most of their energy from oxidizing inorganic electron donors but use organic materials, such as yeast extract, as carbon sources. *Sulfobacillus* spp. are facultative anaerobes that use either molecular oxygen or ferric iron as terminal electron acceptors. In contrast, the genus *Alicyclobacillus* includes species of moderate (pH growth optima 3 - 5) as well as extreme acidophiles. The earliest isolates were obligately heterotrophic moderate thermophiles that were isolated from pasteurised fruit juices which they had contaminated. Later isolates (e.g. *Alb. disulfidooxidans* and *Alb. ferrooxydans*) more resembled *Sulfobacillus* spp., both in terms of the environments they inhabited and in their metabolic capabilities (e.g. in catalysing the dissimilatory oxidation of sulfur and iron). *Alicyclobacillus* spp. tend, however, to be generally more “heterotrophically inclined” than *Sulfobacillus* spp., and grow more successfully using defined organic compounds such as glucose (e.g. [2].

For a number of years, some acidophilic Firmicutes isolated from mineral-rich terrestrial or acidic aquatic environments have been found, from sequencing of their 16S rRNA genes, to be affiliated to neither *Sulfobacillus* nor *Alicyclobacillus* (e.g. [3-5]), though these have not been fully characterized. Clones related to these isolates have also been found in diverse locations (e.g. [6-8]). In this paper, we describe the characteristics of eight such strains, isolated from a variety of low pH environments from different global locations, and show that they comprise two distinct species of a proposed novel genus of Firmicutes, "*Acidibacillus*".

2. Materials and Methods

2.1. Bacterial origins, isolation and cultivation

The eight bacterial strains studied were isolated from a variety of mine-impacted and geothermal sites from different global locations over a 20 year period and maintained within the *Acidophile Culture Collection* at Bangor University (U.K.; Table 1). Bacteria were isolated on solid media that select for the growth of different physiological groups of acidophilic bacteria [9], either directly by streaking water samples (or mine waste slurries) onto plates, or (in the case of isolates Y0010 and ITV01) following enrichment in acidic liquid media. Plates were incubated at either 30º or 45ºC and isolates purified by repeated re-streaking of single colonies onto fresh solid media. These were then transferred into a liquid medium containing 10 mM ferrous sulfate, 0.02% (w/v) yeast extract and acidophile basal salts (ABS), adjusted to pH 2.0 with sulfuric acid. ABS contained (g/L) 0.15 $Na_2SO_4 \cdot 10H_2O$, 0.45 $(NH_4)_2SO_4$, 0.05 KCl, 0.5 $MgSO_4 \cdot 7H_2O$, 0.05 KH_2PO_4, and 0.015 $Ca(NO_3)_2 \cdot 4H_2O$.

2.2. Phylogenetic analysis

DNA was extracted from bacteria grown in 5 mL of ferrous iron/yeast extract liquid medium using the FastDNA *Spin Kit for Soil* (MP Biomedicals) using a modified

protocol [10]. The 16S rRNA genes were amplified from DNA extracts by PCR using DreamTaq PCR Master Mix (Thermo Fisher) and primers 27F (5´-AGT GTT TGA TCC TGG GTC AG-3´; [11]) and GM4R (5´- TAC CTT GTT ACG ACT T-3´; [12]). PCR products were purified, and overlapping sequencing from both sides of the gene was performed by Seqlab (Germany). Contigs were constructed with the software Geneious Pro 5.4, and the resulting gene sequences were analyzed using BLAST at the NCBI database (http://ncbi.nlm.nih.gov/BLAST) and added to the database. Alignment of the sequences obtained, together with those of related strains, was carried out using Mega 6.0 [13], followed by manual editing to remove gaps and positions of ambiguous nucleotides. Phylogenetic trees were constructed by neighbour-joining analyses. Reliability of the tree topologies was confirmed by bootstrap analysis using 1,000 replicate alignments.

2.3. Growth characteristics

Growth rates and optimum pH values and temperatures for growth of the two proposed type strains, SLC66^T ("*A. ferrooxidans*") and Y002^T ("*A. sulfuroxidans*") were determined by growing the bacteria in a pH- and temperature-controlled 2 L bioreactor (Electrolab, UK), as described elsewhere [14]. The liquid medium contained 0.1 mM (SLC66^T) or 1 mM (Y002^T) ferrous sulfate, 0.02% (w/v) yeast extract and ABS, and the bioreactor was stirred at 100 rpm and aerated with ~1.5 L of sterile atmospheric air/minute. Since preliminary experiments had confirmed that growth of both isolates was coupled to ferrous iron oxidation, growth rates were routinely determined from semi-logarithmic plots of ferrous iron oxidized against time.

2.4. Dissimilatory redox transformations of inorganic electron donors and acceptors

Dissimilatory oxidation of ferrous iron was determined by monitoring changes in ferrous iron concentrations and cell numbers in acidic (pH 1.5 to 2.5) liquid medium containing 10 – 25 mM

134 Fe^{2+}, amended (or not) with yeast extract. To determine whether bacteria were able to utilize
135 the energy available from oxidizing iron, strains SLC66^T and Y002^T were grown in replicate
136 flasks containing 0.005% (w/v) yeast extract and different concentrations of ferrous iron (1, 10
137 and 25 mM, at an initial pH of 1.9 for SLC66^T, and 1, 25 and 50 mM, at an initial pH of 1.7 for
138 the more acidophilic isolate Y002^T). Culture media were designed to maximize the amount of
139 ferrous iron oxidation without causing hydrolysis (and precipitation) of the ferric iron generated,
140 which would have impaired the accuracy of cell counts. Cultures were incubated (at 30ºC for
141 SLC66^T and 45ºC for Y002^T), shaken at 100 rpm, and residual ferrous iron and cell numbers
142 determined daily for up to 6 days.

143 Specific rates of ferrous iron oxidation by strain Y002^T grown in ferrous sulfate/yeast extract
144 medium (determined at pH 1.8 and 45ºC) were evaluated as described elsewhere [15]. A
145 modified protocol was used for strain SLC66^T, which involved growing the isolate in a 2 L
146 bioreactor in 25 mM ferrous iron/yeast extract medium (at pH 2.0 and 30ºC) until all of the iron
147 had been oxidized, and then adding a further 3 mM ferrous iron and determining residual Fe^{2+}
148 concentrations over the following 90 minutes. Concentrations of bacterial proteins were
149 measured at the start and end of these experiments to determine whether there had been any
150 significant increase in biomass during the time span of the experiments.

151 Dissimilatory oxidation of elemental sulfur (S^0) was tested by inoculating active cultures into
152 a liquid medium containing ~0.5% (w/v) sterile S^0, with or without 0.02% (w/v) yeast extract,
153 and poised initially at ~pH 3.0. Since the end product of the reaction is sulfuric acid, both
154 changes in pH and sulfate concentrations, as well as increases in cell numbers were used to
155 monitor growth. Oxidation of tetrathionate was assessed by growing isolates Y002^T and Y0010
156 in pH 3 medium that contained 2.5 mM filter-sterilized potassium tetrathionate, 0% or 0.02%
157 yeast extract, 500 µM ferrous iron and ABS. Growth was monitored by enumerating cells and
158 measuring changes in pH and sulfate concentrations.

159 The oxidative dissolution of pyrite by the novel Firmicutes was tested by inoculating the two
160 proposed type strains (SLC66^T and Y002^T) into a liquid medium containing ABS and 1% finely-
161 ground pyrite (Strem Chemicals, USA) supplemented (or not) with yeast extract (0.02%, w/v).

Where yeast extract was not included, cultures were supplemented with trace elements. Replicate shake flask cultures were incubated at either 30ºC (SLC66^T) or 45ºC (Y002^T). Both non-inoculated cultures and others inoculated with the moderate thermophilic Firmicute*, Sb. thermosulfidooxidans*^{T} were incubated in parallel, to act as negative and positive controls (*Sb. thermosulfidooxidans* cultures contained yeast extract and were incubated at 45ºC). Samples were withdrawn at regular intervals to measure pH, redox potential (E_H values), ferrous iron and total soluble iron.

Dissimilatory ferric iron reduction was assessed by growing cultures in 100 mL of liquid medium containing 10 mM ferrous iron, 0.02% yeast extract and ABS, adjusted to either pH 2.0 (isolates SLC66^T, SLC40, ITV01, BSH1, GS1 and Gal-G1; incubated at 30ºC) or 1.8 (isolates Y002^T and Y0010; incubated at 45ºC). The shake flask cultures were incubated, aerobically, until ferrous iron concentrations had fallen to <0.5 mM, at which point 20 mL aliquots were withdrawn from each and placed in 25 mL sterile bottles, further yeast extract added (to 0.02% w/v) and the replicate bottles placed in sealed jars under either anaerobic or micro-aerobic environments (using *AnaeroGen* and *CampyGen* systems, Oxoid, U.K.). Samples were withdrawn after 2 and 4 days (moderate thermophiles) and 10 days (mesophiles) and concentrations of ferrous iron determined. Dissimilatory reduction of sulfur was tested in cultures incubated anaerobically in media containing 10 mM ferrous iron, 0.02% yeast extract, 5 mM glucose and 0.5% elemental sulfur. Growth on hydrogen was tested on solid media using protocols described elsewhere [16].

2.5. Carbon metabolism

Bacteria were grown routinely in liquid medium containing 10 mM ferrous iron and 0.02% (w/v) yeast extract, adjusted to either pH 2.0 (for strains of "*A. ferrooxidans*") or 1.8 (for strains of "*A. sulfuroxidans*"). The effect of adding different concentrations (0, 0.005, 0.02 or 0.5%, w/v) of yeast extract to this medium on cell yields of strains SLC66^T and Y002^T was examined. Comparative growth was also assessed in 1 mM ferrous iron/0.005% yeast extract liquid

medium complimented complex carbon sources (casein hydrolysate and tryptone, both at 0.02%, w/v), or defined organic compounds. The latter were: (i) monosaccharides (glucose, fructose, and maltose, all at 5 mM); (ii) alcohols (15 mM ethanol, 10 mM glycerol and 5 mM mannitol); (iii) organic acids (citric acid and lysine, both at 5 mM); (iv) benzyl alcohol (5 mM). Biomass yields were determined from regular counts of bacteria in liquid media over 3 - 5 days incubation period. Growth yields of all isolates (in triplicate cultures) were also compared using the following liquid media: (i) 1 mM ferrous iron; (ii) 20 mM ferrous iron; (iii) 1 mM ferrous iron/5 mM glucose; (iv) 20 mM ferrous iron/5 mM glucose; (iv) 1 mM ferrous iron/0.005% yeast extract.

2.6. Metal and salt tolerance

Standard ferrous iron/yeast extract liquid medium was supplemented with sterile solutions of aluminum, cobalt, copper, ferrous iron, manganese, nickel or zinc sulfates or sodium molybdate, to give final concentrations of 50 -1000 mM (sulfate salts) or 0.05 - 0.3 mM (molybdate). The pH of the media was adjusted to 2.0 with sulfuric acid and the cultures incubated, shaken, at 30°C ("*A. ferrooxidans*" strains) or 45°C ("*A. sulfuroxidans*" strains) for up to 14 days. Growth was assessed by enumerating bacterial cells. In cultures where ferrous iron tolerance was tested, positive growth was reported by increase in cell numbers determined by SYBR staining [17]. A similar approach to that described above was used to determine salt (sodium chloride) tolerance. Both the highest concentration of metal (or salt) at which growth was observed, and the minimum inhibitory concentrations (MIC) were recorded.

2.7. Genome analyses

Genomic DNA was extracted from cultures of "*A. ferrooxidans*" SLC66^T and ITV01, and from "*A. sulfuroxidans*" Y002^T, grown on 10 mM ferrous iron, 0.02% yeast extract

and 5 mM glucose, using a modified CTAB/high-salt extraction on lysozyme treated cells, followed by alcohol precipitation [18]. Whole-genome sequencing was performed via a combined approach using an Ion Torrent personal genome machine (Life Technologies, Carlsbad, CA), with 400 bp chemistry libraries and 318 semiconductor chip (strain ITV01), and an Illumina MiSeq sequencer with paired-end sequencing kit (strains SLC66^T and Y002^T). Genome assembly was conducted as described elsewhere [19]. Gene sequences coding for proteins in assimilating carbon dioxide and dinitrogen fixation were obtained from the genomes of different acidophilic bacteria: *Acidithiobacillus ferrooxidans* (GCA_000021485.1), *Sulfobacillus acidophilus* (GCA_000219855.1) and *Alicyclobacillus acidocaldarius* (GCA_000024285.1). These were used as query sequences to search the genome scaffolds of the "*Acidibacillus"* spp., employing local Blast with default parameters, using CLC Genomics Workbench 7 (https://www.qiagenbioinformatics.com/). The best hits were investigated as putative orthologs and the protein structures were characterized using InterproScan tools [20].

2.8. Electron microscopy

Active cultures of strains SLC66^T and Y002^T were fixed in 2.5% glutaraldehyde followed by progressive ethanol dehydration. Fixed cultures were filtered through 0,2 µM Nuclepore filters, and the immobilized bacteria critical point-dried and gold-coated, and were visualized using a Zeiss Sigma VP scanning electron microscope.

2.9. Analytical methods and reference bacteria

Bacteria were enumerated using a Helber counting chamber marked with Thoma ruling (Hawksley, United Kingdom) and viewed with a Leitz Labolux phase-contrast microscope at a magnification of 400X. Ferrous iron was determined using the Ferrozine reagent [21]. Total soluble iron was determined using the same method but

following reduction of ferric iron to ferrous by adding ascorbic acid, and ferric iron concentration from differences in the two values. Protein concentrations were measured using the Bradford assay [22]. Concentrations of glucose were determined using a Dionex ICS 3000 ion chromatography system fitted with a Carbo Pac MA1 column and ED amperometric detector, and sulfate concentrations using a Dionex IC25 ion chromatograph with an Ion Pac AS-11 column equipped with a conductivity detector. Culture pH was measured using a pHase combination glass electrode, and redox potentials (adjusted to be relative to a standard hydrogen electrode; E_H values) using a combination platinum silver/silver chloride electrode (VWR, UK). Both electrodes were coupled to an Accumet pH/redox meter 50.

The type strain of *Sb. thermosulfidooxidans* (DSM 9293) and *Acidiphilium cryptum* strain SJH [9] were used in some experiments

3. Results

3.1. Bacterial cells and colonies

All eight novel bacteria were isolated on acidic overlay solid media that contained organic carbon (tryptone soya broth or yeast extract) in addition to ferrous iron. They were subsequently subcultured on "FeSo" medium [9] which contains 2.5 mM potassium tetrathionate in addition to ferrous iron and tryptone soya broth. Bacterial colonies on FeSo medium had "fried egg" morphologies (Supplementary Fig. 1) typical of heterotrophic iron-oxidizing acidophiles, the orange coloration of the colony centres resulting from the accumulation of oxidized iron. Cells of "*A. ferrooxidans*" SLC66^T were motile rods, 1.5 – 1.8 µm long, ~0.4 µm wide, and formed oval endospores which were located at the cell termini. Cells of "*A. sulfuroxidans*" Y002^T were also motile rods, 3 - 4 µm by ~0.5 µm that formed oval endospores located at the cell termini. It was noted that numbers of individual cells of Y002^T increased during the early phases of

incubation (up to 2 days) but declined subsequently; this appeared to be related to cells aggregating as incubation progressed (Supplementary Fig. 2), a feature that was much less apparent in cultures of SLC66^T.

3.2. Phylogenetic analysis

Analysis and comparison of 16S rRNA gene sequences confirmed that all eight isolates were members of the phylum Firmicutes (order *Bacillales*, family *Alicyclobacillaceae*). Figure 1 shows that they clustered into three closely-related groups, two of which (Groups IA and IB) shared >99% similarity of their 16S rRNA genes and all of these were proposed to be strains of the novel species "*A. ferrooxidans*". The two Group II isolates (Y002^T and Y0010) shared >99% 16S rRNA gene similarity but were more distantly related (94% gene similarity) to both Groups IA and IB and considered to be strains of a different species, "*A. sulfuroxidans*". These phylogenetic differences were also reflected in some key physiological traits, described below.

3.3. Effects of pH and temperature on growth rates

Figure 2 shows the effects of pH and temperature on the culture doubling times (t_d's) of SLC66^T and Y002^T. Both bacteria were confirmed to be extreme acidophiles, though Y002^T was more acidophilic with a pH optimum and minimum for growth of 1.8 and 1.6, respectively, while corresponding values for SLC66^T were 2.9 and 1.9. The two isolates also displayed contrasting temperature-related growth: SLC66^T was mesophilic (temperature optimum and maximum of ~30ºC and 37.5ºC) while Y002^T was a moderate thermophile with an optimum growth temperature of ~43ºC and a maximum of 50ºC. When grown at optimum conditions of pH and temperature, SLC66^T had a culture doubling time (t_d) of 6.7 h (corresponding to a growth rate, μ, of 0.10 h$^-$

302 ^{1}), while the moderate thermophile Y002^{T} grew much more rapidly (minimum t_d of 2.1
303 h, corresponding to a μ_{max} of 0.33 h^{-1}). Tests carried out in shake flasks confirmed that
304 none of the "Group I" isolates ("*A. ferrooxidans*") grew at pH 1.5, though one Group IA
305 strain (BSH1) and both Group IB strains (ITV01 and Gal-G1) grew at pH 1.75
306 (Supplementary Table 1). None of the four Group IA strains grew at 40ºC, in contrast
307 to both Group IB strains (though neither of these grew at 45ºC). Like Y002^{T}, the other
308 strain of "*A. sulfuroxidans*" (strain Y0010) grew at 45ºC, though this bacterium was less
309 acidophilic than Y002^{T} and grew at pH 1.75 though not at pH 1.5 (Supplementary Table
310 1).
311
312 *3.4. Dissimilatory redox transformations of inorganic electron donors and acceptors*
313
314 All eight of the isolates catalysed the oxidation of ferrous iron in acidic media, and
315 this was found to be highly correlated (r^2 = 0.98 - 0.99) with growth of the bacteria. In
316 cultures containing very small concentrations (0.005% w/v) of yeast extract, cell yields
317 of both SLC66^{T} and Y002^{T} increased in parallel with the amount of ferrous iron oxidized
318 (Fig. 3a). However, this trend was not found in cultures that contained a much higher
319 (0.05%) concentration of yeast extract, and in these cell numbers were also noted to
320 continue to increase beyond the point at which all of the ferrous iron had been oxidized
321 (Supplementary Fig. 3). The specific rates of ferrous iron oxidation were 36.1 +/- 3.4
322 mg min^{-1} mg $protein^{-1}$ (SLC66^{T}, at 30ºC and pH 2.0) and 48.5 +/- 1.3 mg min^{-1} mg
323 $protein^{-1}$ (Y002^{T}, at 45ºC and pH 1.8).
324 None of the six Group I ("*A. ferrooxidans*") strains oxidized elemental sulfur. In
325 contrast, pH declined and sulfate concentrations increased as a result of the
326 dissimilatory oxidation of sulfur to sulfuric acid, in yeast extract-containing cultures of
327 both Group II isolates (Y002^{T} and Y0010; Supplementary Fig. 4). Numbers of Y002^{T}
328 and Y0010 did not, however, correlate with oxidation of sulfur, which was considered
329 to be due to attachment of cells to particulate S^0. In contrast, numbers of both Y002^{T}

and Y0010 increased in tetrathionate-containing media, paralleling changes in sulfate concentrations and culture pH (and was more pronounced in cultures of Y002^T), confirming that the two strains of "*A. sulfuroxidans*" can oxidize tetrathionate as well as elemental sulfur.

"*A. ferrooxidans*" SLC66^T catalysed the oxidative dissolution of pyrite, as evidenced by increasing concentrations of total soluble iron and cultures developing more positive E_H values with incubation time, though again this was only observed with yeast extract-containing cultures (Fig. 4a). Cultures of Y002^T, in contrast, initially failed to show any evidence of pyrite oxidation in liquid media that had been prepared under identical conditions. It was also noted that this isolate was unable to oxidize the ferrous iron released during sterilization of pyrite when the mineral was autoclaved in the presence of yeast extract. However, when sterile yeast extract solution was added subsequent to autoclaving pyrite/ABS, oxidative dissolution of pyrite proceeded, as shown in Fig. 4b. As with SLC66^T, it was found that pyrite oxidation by strain Y002^T was negligible in yeast extract-free medium, though adding yeast extract to "inorganic" cultures of Y002^T at day 12 resulted in rapid oxidation of the ferrous iron present, as indicated by a mean increase in redox potential of >200 mV during the following two days, and the initiation of pyrite dissolution (Fig. 4b). The addition of further yeast extract (at day 12) to cultures of Y002^T that had yeast extract added at the start of the experiment also resulted in more positive E_H and accelerated pyrite oxidation. Pyrite dissolution by "*A. sulfuroxidans*" Y002^T was noted to be about 40% less extensive than that observed in cultures of *Sb. thermosulfidooxidans*^{T} grown under identical conditions.

All strains of "*Acidibacillus*" tested were able to catalyse the dissimilatory reduction of ferric iron under anaerobic conditions (Supplementary Fig. 5), though no reduction was observed in parallel cultures incubated under micro-aerobic conditions (data not shown). The two strains of "*A. sulfuroxidans*" displayed the greatest propensity for iron reduction, and the two Group IB strains the least. None of the isolates was found to

358 reduce elemental sulfur or catalyse the dissimilatory oxidation of elemental hydrogen.
359 Cultures on solid media grown in H_2-enriched or H_2-free atmospheres were identical in
360 size and morphology after protracted incubation, in contrast to those of the positive
361 control acidophile, *Sb. thermosulfidooxidans*.
362
363 *3.5. Utilization of organic carbon*
364
365 All of the novel isolates required a source of organic carbon for growth in liquid
366 media, and yeast extract appeared to be superior to all others tested for this purpose.
367 Biomass yields of both SLC66^T and Y002^T correlated with concentrations of yeast
368 extract (Fig. 3b), though cell numbers of SLC66^T were mostly much greater than those
369 in the equivalent cultures of Y002^T, though this was at least partially due to more
370 pronounced cell aggregation of the latter, as noted previously.
371 Addition of some complex and defined organic compounds to ferrous iron/yeast
372 extract liquid media resulted in increased cell numbers of all of the "*Acidibacillus*"
373 strains, though this was limited in scale (Supplementary Table 2). Comparative data
374 for the mesophilic acidophiles SLC66^T and *Acidiphilium* SJH (Fig. 5) show that
375 numbers of the former were far fewer than those of *Acidiphilium* SJH, grown in identical
376 glucose-containing liquid medium. Also, while all of the glucose provided was utilized
377 in the *Acidiphilium* SJH cultures within 3 days, only ~12% of the glucose in cultures of
378 SLC66^T was metabolized. In the case of Y002^T, numbers were >50% greater in cultures
379 containing glucose than in glucose-free controls, but only ~6% of the available glucose
380 was utilized (data not shown). In the case of strain Y002^T, cell numbers were also
381 significantly greater (by ~55% on day 3 and ~500% on day 7) in iron/yeast extract
382 cultures that contained glucose compared to those that did not, though again the
383 amount of glucose consumed was relatively small (5.5% of that provided;
384 concentrations, compensated for evaporative water loss, falling from 4.90 +/- 0.02 mM
385 on day 0 to 4.62 +/- 0.06 on day 7).

Figure 6 compares numbers of all eight isolates grown in different inorganic and organic-amended liquid media. While there were some differences displayed between the "*Acidibacillus*" strains, there were some interesting general trends, including the observation that greater amounts of ferrous iron did not generally result in enhanced cell yields in organic carbon-free media, though they did in most cases where glucose was also present. Cell numbers of all of the strains were also much greater in 1 mM ferrous iron medium containing 0.005% yeast extract than in those containing 5 mM glucose, even though the amount of organic carbon present (~ 25 mg/L, compared to 360 mg/L) was much less in the former.

3.6. Tolerance of "Acidibacillus" spp. to some transition metals, aluminium and sodium chloride

The tolerance of the six strains of "*A. ferrooxidans*" and the two strains of "*A. sulfuroxidans*" to aluminum and selected transition metals are shown in Table 2. Strains belonging to "*A. ferrooxidans*" had in general a higher tolerance towards most of the metals tested than the two "*A. sulfuroxidans*" isolates. The proposed type stain of "*A. ferrooxidans*" (SLC66) had a lower MIC for copper than the other strains belonging to the same species. Strain BSH1 displayed less tolerance of copper but had by far a higher tolerance threshold for cobalt than other strains of this species. Strains of "*A. sulfuroxidans*" were far more sensitive to copper and cobalt than the two "*A. ferrooxidans*" strains None of the isolates were halotolerant, though both strains of "*A. sulfuroxidans*" were able to grow in liquid media containing higher concentrations of sodium chloride than the six strains of "*A. ferrooxidans*" tested (Table 2).

3.7. Genome compositions, and carbon- and nitrogen-fixation genes

Data from the preliminary annotation of the genomes of the three strains of "*Acidibacillus*" (SLC66^T, ITV01 and Y002^T) showed that they had GC contents of 52%, 50% and 46%, respectively. The current assemblies contain 3.03 Mbp for SLC66^T, 3.23 Mbp for ITV01 and 2.70 Mbp for Y002^T. The genomes of strains SLC66^T and ITV01 contained genes with relatively low (34% and 36%, respectively) similarity to the *cbbL* gene (which encodes the large subunit of RuBisCO form IA, involved in CO_2 assimilation) but not the *cbbM* gene (which encodes the large subunit for type II RuBisCO). No gene identified as being necessary for nitrogen fixation were identified in the three genomes, but BLAST searches revealed a low sequence identity (36%) for the gene encoding for rusticyanin (a protein involved in ferrous iron oxidation in the iron-oxidizing acidithiobacilli and some other acidophiles) in the genomes of the three "*Acidibacillus*" strains.

4. Discussion

The bacteria described in this report were isolated from geothermal and mine-impacted sites from different parts of the world. The fact that other closely related acidophiles have also been isolated from sites in Germany [4, 5] and clones identified in samples in China [6], Japan [7] and Argentina [8] suggests that "*Acidibacillus*" spp. are very widely distributed in extremely acidic environments. The first reported strains ("SLC series") were all described as obligately heterotrophic, mesophilic iron-oxidizing acidophiles, and were noted to be only distantly phylogenetically related to other Firmicutes [3]. Six other phylogenetically-related isolates that have since then been added to the *Acidophile Culture Collection* at Bangor University since then were studied alongside two of the original "SLC series" strains in the present study. While the eight strains shared a number of physiological traits, there were also some significant differences.

441 Comparison of 16S rRNA gene sequences clearly separated the eight strains
442 studied, at the genus level, from currently classified acidophilic Firmicutes. While they
443 formed a distinct clade, the fact that two of the isolates (Y002^T and Y0010) shared
444 only 94% gene similarity with the other six confirmed that the isolates comprised two
445 distinct species. Subsequent laboratory tests showed that these phylogenetic
446 relationships were reflected in some marked differences in some key physiological
447 characteristics, with the larger group being mesophilic iron-oxidizers ("*A.*
448 *ferrooxidans*") and the smaller group moderately thermophilic iron- and sulfur-
449 oxidizers that were more tolerant of extreme acidity ("*A. sulfuroxidans*"). Interestingly,
450 phylogenetic analysis separated two strains of the larger group (strains ITV01 and
451 Gal-G1) from the other four strains, even though the six strains shared ~99% gene
452 similarity, and this was also reflected in some minor differences in their physiologies.
453 For example, strains ITV01 and Gal-G1 grew at 40ºC, while the other four strains of
454 "*A. ferrooxidans*" did not, both grew at pH 1.75 while only one Group IA strain (BSH1)
455 grew at this pH value, and strains ITV01 and Gal-G1 were also the least effective of
456 all eight strains at reducing ferric iron.
457 The three major physiological traits shared by all of the isolates studied were: (i)
458 optimum growth at extremely low (<3) pH, (ii) the ability to catalyse the dissimilatory
459 oxidation of ferrous iron, and (iii) a requirement of organic carbon for growth. In
460 contrast to *Sulfobacillus* spp. [16], none of the isolates used molecular hydrogen as
461 an energy source. All of the isolates also catalysed the dissimilatory reduction of
462 ferric iron under anoxic conditions though, as noted, this was limited in the case of
463 the two Group IB strains, and it was not ascertained whether the bacteria could grow
464 by ferric iron respiration. Ferrous iron is a widely used electron donor among
465 acidophilic prokaryotes, due to it often being present in large concentrations in low
466 pH environments, and also chemically stable at pH < 3 [1]. The ability to oxidize
467 ferrous iron does not necessarily imply that microorganisms are able to conserve the
468 energy from this reaction. However, the observation that cell numbers of both

SLC66^T and Y002^T increased in parallel with the amount of iron oxidized (in organic-lean media) strongly suggests that this is the case with "*Acidibacillus*" spp.. The specific rates of ferrous iron oxidation recorded for "*Acidibacillus*" spp. (36.1 +/- 3.4 mg min^{-1} mg protein^{-1} for SLC66^T, and 48.5 +/- 1.3 mg min^{-1} mg protein^{-1} for Y002^T) were much lower than those reported for other oxidizing acidophiles (192 – 484 mg min^{-1} mg protein^{-1} for chemolithotrophic *Leptospirillum* and *Acidithiobacillus* spp., and 236 – 449 mg min^{-1} mg protein^{-1} for *Sulfobacillus* spp. [15]).

The two "*A. sulfuroxidans*" strains (Y002^T and Y0010) also catalysed the dissimilatory oxidation of both elemental sulfur and tetrathionate, and it was assumed (though not confirmed) that they also conserved the energy from these reactions. The ability to oxidize both ferrous iron and sulfur is not uncommon among chemolitho-autotrophic and chemolitho-heterotrophic acidophiles, and has been reported for some *Acidithiobacillus* spp. (*At. ferrooxidans, At. ferridurans, At. ferrivorans* and *At ferriphilus*. [26]), *Acidihalobacter prosperus* [27], *Acidiferribacter thiooxydans* [28] and "*Acidithiomicrobium*" [29]. Among the acidophilic Firmicutes, all classified *Sulfobacillus* spp. (*Sb. thermosulfidooxidans, Sb. acidophilus, Sb. thermotolerans, Sb. benefaciens,* and *Sb. sibiricus*) [1], as well as *Alicyclobacillus tolerans* and *Alb. aeris* [30] can oxidize both ferrous iron and reduced sulfur. Other species of acidophilic bacteria (e.g. *Leptospirillum ferrooxidans, Ferrimicrobium acidiphilum, Acidimicrobium ferrooxidans, Acidithrix ferrooxidans* and "*Ferrovum myxofaciens*") catalyse the dissimilatory oxidation of ferrous iron but not sulfur [1] as was the case with the six strains of "*A. ferrooxidans*".

Yeast extract acted as both an energy and carbon source for these bacteria, as evidenced by: (i) growth continuing in cultures well after all of the ferrous iron had been depleted, (ii) growth yields correlating with concentrations of yeast extract provided (in cultures containing relatively little ferrous iron) and (iii) active growth in yeast extract/ferric iron media. "*Acidibacillus*" spp. can therefore be classified as facultative chemolitho-heterotrophs (i.e. they can obtain energy from both inorganic

497 and organic electron donors but require an organic carbon source). It was noted that
498 cell yields of "*A. ferrooxidans*" SLC66^T tended to be greater than those of "*A.*
499 *sulfuroxidans*" Y002^T in liquid media that contained the same concentrations of yeast
500 extract. This was thought to be due, at least in part, to strain SLC66^T being able to
501 utilize a wider range of organic compounds present in this complex material, as both
502 carbon and energy sources, than strain Y002^T. Cell yields of both SLC66^T and Y002^T
503 were significantly greater when glucose was added to ferrous iron/yeast medium,
504 suggesting that it was metabolized to some extent. In contrast to those of the
505 heterotrophic acidophile, *Acidiphilium* SJH, only small amounts (5.5 - 6%) of the
506 available glucose was utilized in these cultures, suggesting that this compound
507 served as a carbon source, but not an energy source, for "*Acidibacillus*" spp., and
508 that growth was ultimately limited by the energy source available (ferrous iron, and
509 that fraction of yeast extract that could be broken down to generate ATP) in these
510 cultures. Glucose could also act as a carbon source for "*Acidibacillus*", but was far
511 less effective than yeast extract. For most strains, growth yields in glucose-containing
512 media was limited by the availability of ferrous iron, suggesting again that the later
513 served as the sole or main energy source, and glucose as the carbon source for
514 these bacteria. Further annotation of the genomes of these bacteria will undoubtedly
515 throw more light on the biochemical constraints that restrict glucose utilization by
516 these novel acidophiles. The information available so far confirms that "*Acidibacillus*"
517 spp. are not diazotrophic. Genes with relatively low sequence identity to *ccbL* (though
518 not *cbbM*) gene were found in the genomes of two of the three sequenced bacteria,
519 though extensive laboratory tests confirmed that none of the strains could grow in the
520 absence of an organic form of carbon. Intriguing is the fact that all three bacteria
521 appear to contain a gene that is related to that which encodes for rusticyanin, a
522 protein known to be involved in iron oxidation in *Acidithiobacillus* spp. but not, so far,
523 in the iron-oxidizing Firmicutes.

In theory, any acidophilic bacterium that catalyses the dissimilatory oxidation of ferrous iron should accelerate the oxidative dissolution of pyrite, as ferric iron is the primary oxidant of this mineral in acidic liquors [31]. This was the case with both "*Acidibacillus*" type species, though it was not immediately apparent for strain Y002^T. Autoclaving pyrite in the presence of yeast extract (as is common practice in the Bangor research laboratories, and has not previously proven problematic) generated some, currently unidentified, by-product that inhibited growth and iron oxidation by strain Y002^T, though not by strain SLC66^T and the positive control Firmicute, *Sb. thermosulfidooxidans*. Adding sterile yeast extract after heat-sterilization of pyrite eliminated this impediment, though pyrite leaching by Y002^T was far less effective, and appeared to require more yeast extract, than that by *Sb. thermosulfidooxidans*.

The major industrial use of iron- and sulfur-oxidizing acidophilic bacteria is in the commercial bio-processing of sulfide mineral ores to extract and recover base and precious metals ("biomining" [32]). Whether or not "*Acidibacillus*" spp. have a potential role in mineral bioleaching consortia has yet to be evaluated. Both species could, in theory, carry out two critical roles (those of regenerating ferric iron and removing potentially inhibitory organic carbon) and "*A. sulfuroxidans*" strains could also contribute to the process by generating sulfuric acid. Another important required characteristic – that of being able to tolerate highly elevated concentrations of transition and other metals – also appears to be adequate, as the data obtained showed that metal tolerance is similar to that of most of the iron-oxidizing *Acidithiobacillus* spp.. A more significant constraint, however, may be their tolerance to extreme acidity, as many biomining practices operate at pH values <2, and often (in stirred tanks) at ~ pH 1.5. Mesophilic "*A. ferrooxidans*" may, however, play a more important role in the natural attenuation of acidic (pH >2) ferruginous mine waters by catalysing the oxidation of ferrous iron and thereby facilitating the hydrolysis and precipitation of ferric iron [33].

Conflict of interest

There are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://xxxx

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661

662 Table 1

663 Sites of origin of bacteria identified as *Acidibacillus* spp..

Isolate	Origins and dates of isolation	Reference
SLC66^T & SLC40	Experimental system used to accelerate the oxidation of mine waste (pH 2.9, 25°C); Utah (1994)	[3]
Gal-G1	Geothermal area (pH 3.0, 80°C); Soufriere Hills, Montserrat, W.I. (1996)	[23]
Y002^T & Y0010	Geothermal area (pH 2.7, 30-60°C) Yellowstone National Park, Wyoming (2000)	[24]
ITV01	Stream draining waste rock at a copper mine (pH 4.9, 32°C); Brazil (2013)	I. Ñancucheo et al. (unpublished)
BSH1	Constructed wetland receiving coal mine drainage (pH 7.0, 14°C); England (2014)	C. Falagan et al. (unpublished)
GS1	Sediment in a pit lake at an abandoned copper mine (pH 3.8, 23°C); Spain (2015)	[25]

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665

Table 2

Tolerance of strains of "*Acidibacillus*" to selected metals and chloride. The values (in mM) shown are minimum inhibitory concentrations (MICs) and those in parentheses are the highest concentrations of that metals/chloride where growth was observed.

	Cu	**Zn**	**Ni**	**Co**	**Al**	**Mn**	**Fe(II)**	**Mo**	**Cl**
SLC66^T	300 (200)	1000 (800)	400 (200)	300 (200)	800 (600)	>800	800 (600)	<0.05	100 (50)
SLC40	600 (400)	1000 (800)	400 (200)	300 (200)	800 (600)	>800	600 (400)	<0.05	50 (25)
BSH1	400 (200)	1000 (800)	400 (200)	>600	1000 (800)	800 (600)	800 (600)	<0.05	100 (50)
GS1	800 (600)	800 (600)	400 (200)	300 (200)	600 (400)	800 (600)	600 (400)	<0.05	100 (50)
ITV01	600 (400)	1000 (800)	400 (200)	300 (200)	800 (600)	>800	800 (600)	<0.05	100 (50)
G1	800 (600)	600 (400)	400 (200)	300 (200)	800 (600)	600 (400)	800 (600)	<0.05	50 (25)
Y002^T	100 (50)	200 (100)	200 (100)	50 (30)	400(300)	800 (600)	600 (400)	<0.05	250 (100)
Y0010	300 (200)	200 (100)	300 (200)	150 (100)	600 (400)	400 (300)	600 (400)	<0.05	250 (100)

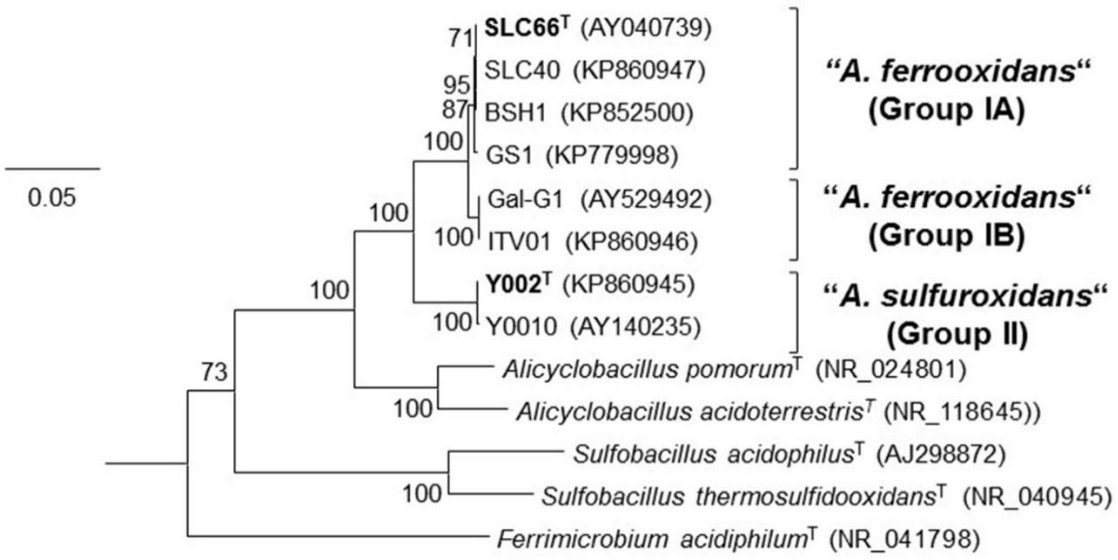

Fig. 1. Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequence data showing the relationship of "*Acidibacillus ferrooxidans" and "A. sulfuroxidans"* to other Gram-positive acidophiles. GenBank accession numbers are given in parentheses for each strain. The tree was rooted with *Acidianus brierleyi*^{T} (not shown). Bootstrap values are given at the respective nodes.

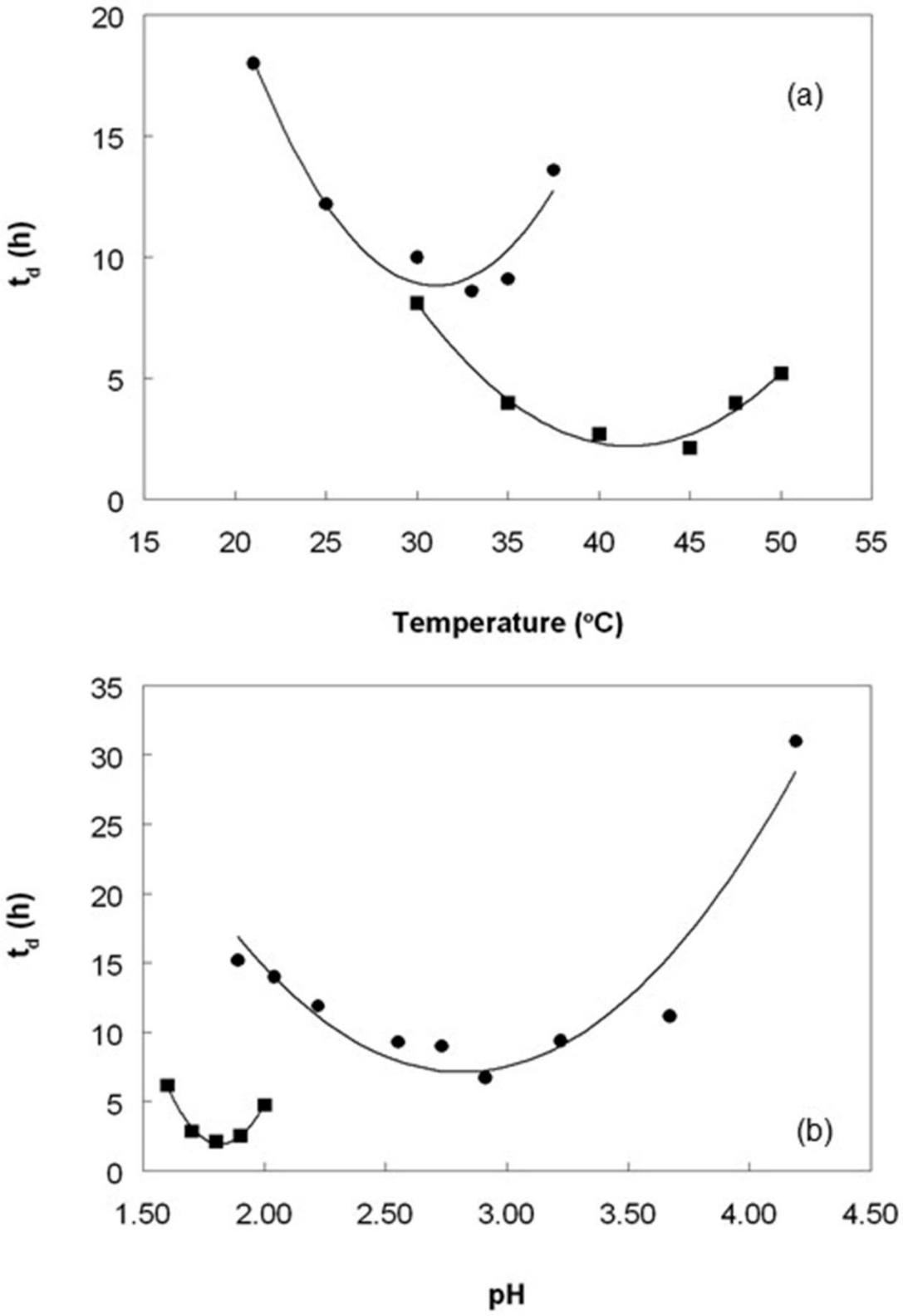

Fig. 2. Effects of (a) temperature and (b) pH on culture doubling times of "*A. ferrooxidans*" SLC66^T (●) and "*A. sulfuroxidans*" Y002^T (■).

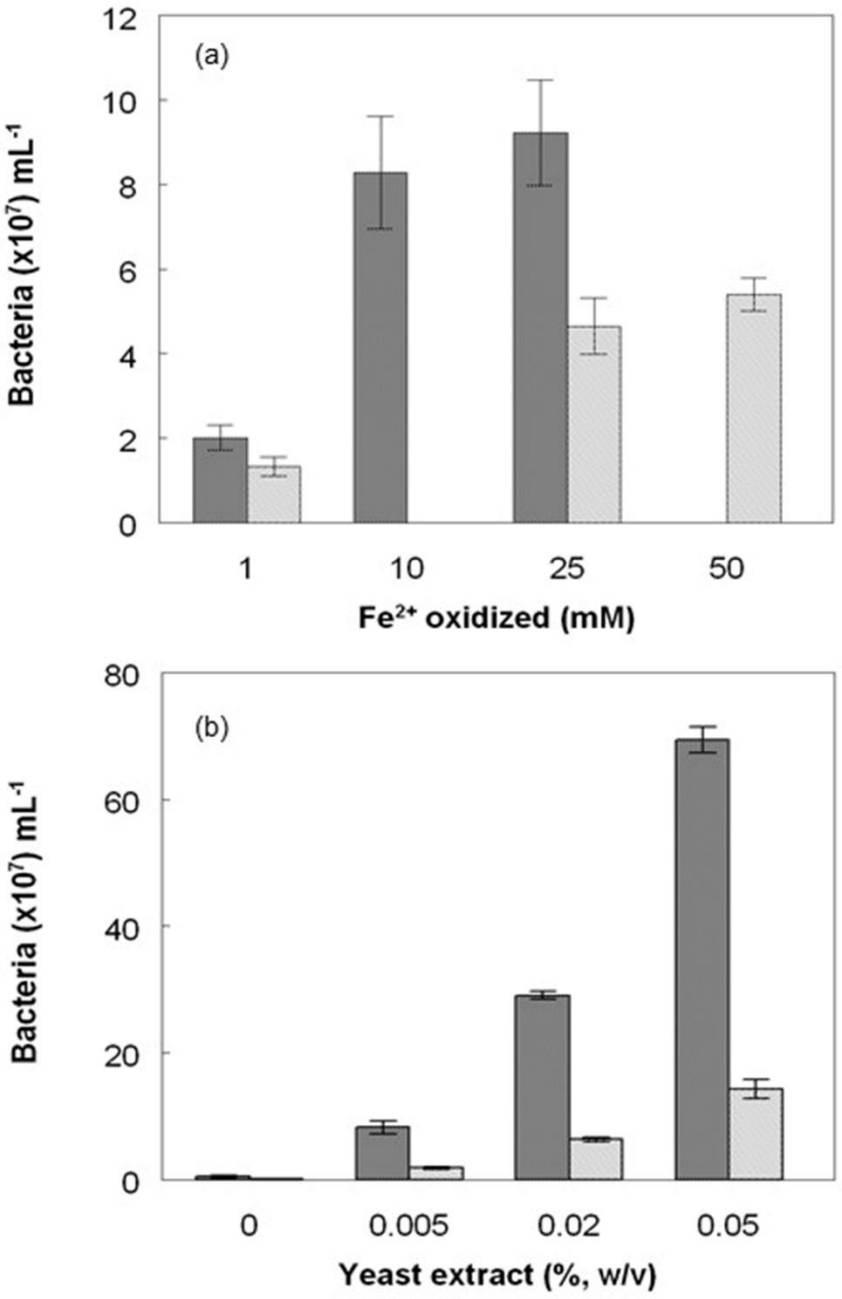

Fig. 3. (a) Effect of ferrous iron concentrations, and (b) yeast extract concentrations on cell numbers of *A. ferrooxidans* SLC66^T (dark shaded bars) and *A. sulfuroxidans* Y002^T (light shaded bars). Cultures were grown either at a fixed (0.005%, w/v) yeast extract concentration and variable amounts of ferrous iron and cells counted when all of the iron had been oxidized (a), or at a fixed (10 mM) ferrous iron concentration and variable amounts of yeast extract and maximum cell numbers (found after 2 days incubation in cultures of Y002^T, and 4 days in cultures of SLC66^T) recorded. Bars indicate mean values and the error bars data range (n = 2).

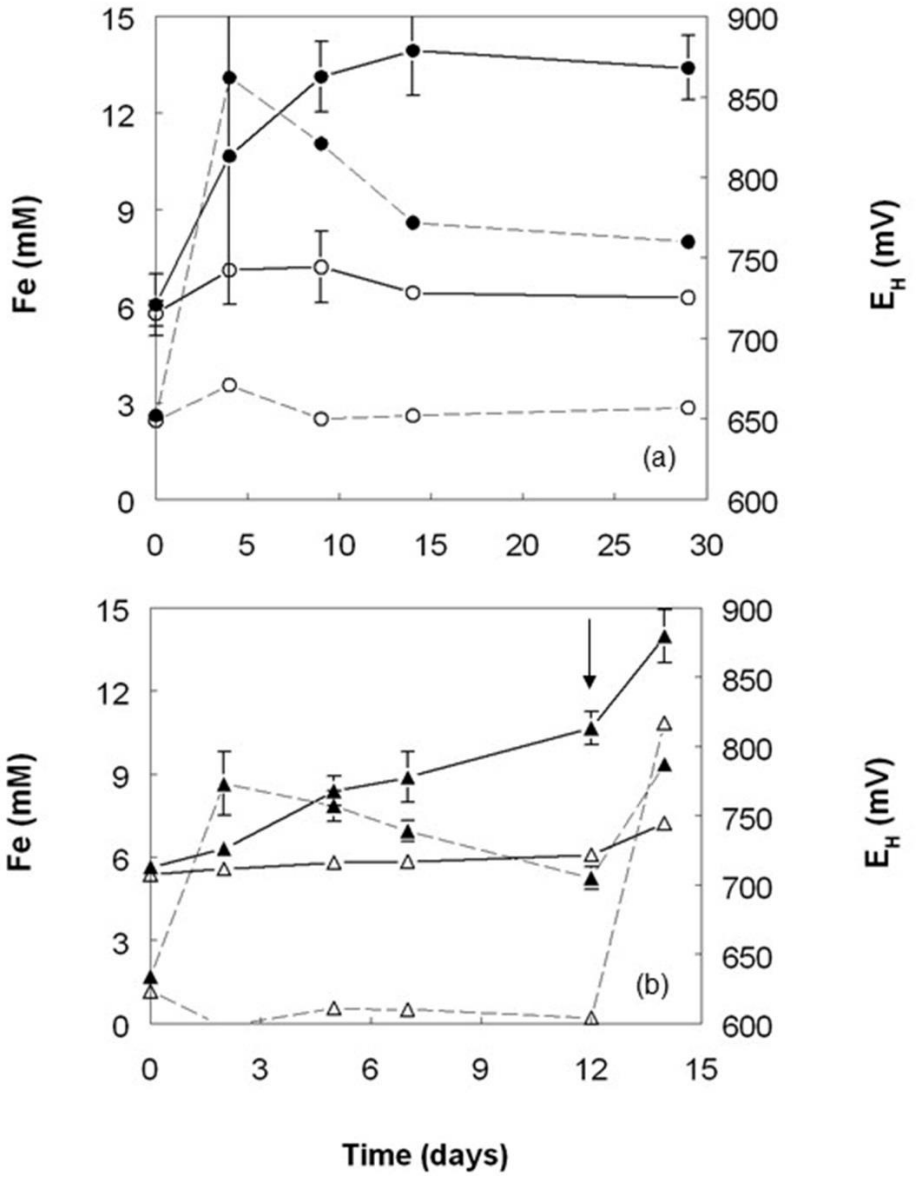

Fig. 4. Oxidative dissolution of pyrite by (a) "*A. ferrooxidans*" SLC66^T (at 30ºC) and (b) "*A. sulfuroxidans*" Y002^T (at 45ºC) in the presence and absence of 0.02% yeast extract. Key: SLC66^T with (●) and without (o) yeast extract; Y002^T with (▲) and without (Δ) yeast extract. Solid lines show total soluble iron concentrations and broken lines redox potentials (E_H) values (symbols show mean values and error bars range values of replicate cultures). The arrow in (b) shows the point at which (day 12) sterile yeast extract was added to both the yeast extract-free and yeast extract-containing cultures. Non-inoculated control cultures showed little change in total soluble iron and redox potential during the time course of the experiments. Data points indicate mean values and error bars data range (n = 2).

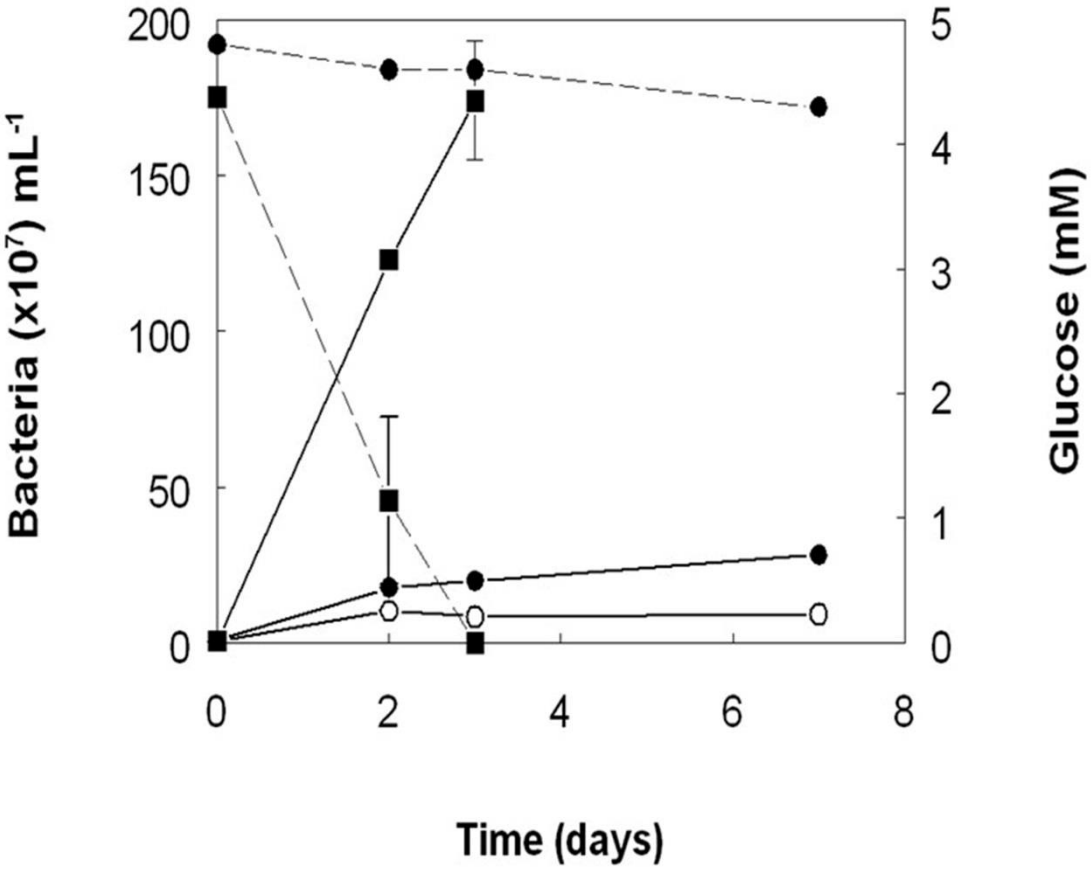

Fig. 5. Comparison of changes in cell numbers (solid lines) and glucose concentrations (broken lines) in cultures of "*A. ferrooxidans*" SLC66^T and *Acidiphilium* SJH. Key: cell numbers and glucose concentrations in cultures of "*A. ferrooxidans*" SLC66^T grown with (●) or without (o) 5 mM glucose; cell numbers and glucose concentrations in cultures of *Acidiphilium* SJH grown with 5 mM glucose (■).Data points indicate mean values and error bars data range (n = 2).

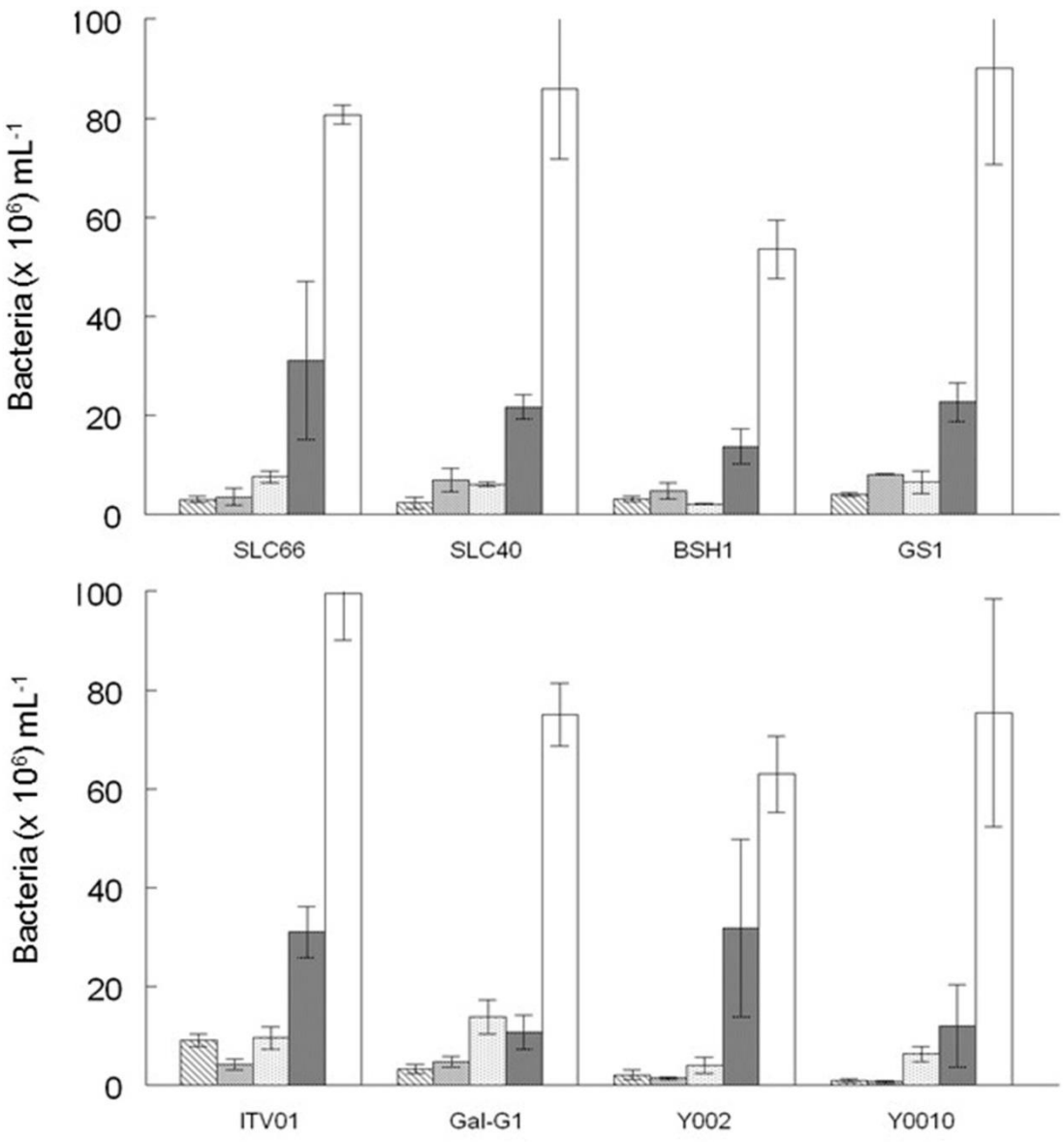

Fig. 6. Cell counts of "*Acidibacillus*" spp. grown in different liquid media; Key: 1 mM Fe^{2+}; 20 mM Fe^{2+}; 1 mM Fe^{2+}/5mM glucose; 20 mM Fe^{2+}/5 mM glucose; 1 mM Fe^{2+}/0.005% yeast extract. Bars indicate mean values and error bars standard deviations (n = 3).